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GRANT NUMBER DAMD17-96-1-6176

TITLE: Genetic Elements for Chemoprotection Against Cyclophosphamide

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (19 Aug 96-18 Aug 97)

4. TITLE AND SUBTITLE
Genetic Elements for Chemoprotection Against Cyclophosphamide

5. FUNDING NUMBERS
DAMD17-96-1-6176

6. AUTHOR(S)
Dr. Victor Levenson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Our goal is identification of genes that affect cellular response to alkylating agent cyclophosphamide (CP); expression modulation of such genes will protect cells from CP toxicity.

Chemotherapeutic approach to advanced breast cancer treatment relies heavily on the use of CP. High-dose CP therapy has to deal with several dangerous side effects, myelosuppression being the most serious. Autologous bone marrow transplantation (ABMT) or peripheral blood stem cell transplantation (PBSCT) is an effective adjunct therapy. This approach, however, has several serious drawbacks: (1) initial myelosuppression with acute leukopenia and increased risk of infectious complications; (2) possibile contamination with neoplastic cells increases with every consecutive round of transplantation; (3) escalating costs of successive rounds of ABMT/PBSCT.

We will identify genetic elements which protect cells from toxic effects of CP; these elements, when introduced in bone marrow in the course of the first round of ABMT/PBSCT, would protect bone marrow from CP. ABMT/PBSCT with CP-resistant bone marrow (1) will allow dose escalation without hematological complications; (2) will reduce the risk of re-introduction of neoplastic cells; and (3) will reduce the economic impact of the disease. Identification of genes involved in CP resistance will provide insights into mechanisms of this process.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Breast Cancer	Cyclophosphamide Chemotherapy Gene therapy	17
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Victor Lewison 9-18-97

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Introduction.

Subject.

Identification of genetic mechanisms which determine cellular resistance to chemotherapeutic agent cyclophosphamide.

Purpose.

To identify genes that affect cellular response to alkylating agent cyclophosphamide (CP) and its derivatives; expression modulation of such genes will protect cells from CP toxicity.

Scope.

The proposed research will identify genetic elements which protect cells from toxic effects of CP; these elements, when introduced in bone marrow in the course of the first round of autologous bone marrow transplantation/peripheral blood stem cell transplantation (ABMT/PBSCT), would protect bone marrow from CP, thus reducing or eliminating myelosuppressive effects of this drug and making additional rounds of ABMT/PBSCT unnecessary. ABMT/PBSCT with genetically modified, CP-resistant bone marrow (1) will allow rapid dose escalation without the risk of hematological complications; (2) will reduce the risk of re-introduction of neoplastic cells, imminent with repeated rounds of ABMT/PBSCT, and (3) significantly reduce the economic impact of the disease. Additionally, identification of genes involved in CP resistance will provide important insights into mechanisms of CP cytotoxicity and resistance and may also suggest new approaches for enhancing the antitumor effect of CP.

Background

Advanced breast cancer is currently associated with extremely poor prognosis when both hormone treatment and chemotherapy are considered palliative rather than curative treatments (reviewed in 1). Hormone therapy is the first line of defense, and it can be used if cancer cells retain functional estrogen and progesterone receptors and visceral tumor nodes are absent (2). However, a rapid decrease in the response rate to hormonal therapy ultimately necessitating chemotherapy, usually with a combination of several drugs. The standard chemotherapeutic regimens for breast cancer are based on doxorubicin (often used as a single agent) and cyclophosphamide-methotrexate-5'-fluorouracyl (CMF), which consists of an alkylating agent cyclophosphamide, 5-fluorouracil, which inhibits thymidilate synthase, and dihydrofolate reductase inhibitor methotrexate (3). This well-established regimen is sometimes augmented by mitoxantrone (4), leucovorin (5), thiotepa (6), pentoxifylline (7), etoposide (8), etc., but these compounds are used much less frequently, and their ability to replace the agents of the CMF regimen is still under investigation (see 9 for a review).

Cyclophosphamide - an alkylating agent from oxazaphosphorine family - requires activation by hepatic microsomal enzymes; its active form - 4-hydroxycyclophosphamide (4-HC) - is released in blood stream and transported throughout the body. Entering the cell 4-HC is converted into aldophosphamide which decomposes into phosphoramide mustard (PM) and acrolein. Alkylation of DNA by PM - guanine at position O^6 or adenine at position 3 - induces either point mutations (in case of O^6-alkylguanine, 10) or DNA breaks through apurinic/apyrimidinic sites (in case of 3-alkyladenine, 11).

PM can also induce inter- and intrastrand DNA crosslinks as well as DNA-protein crosslinks (12) while acrolein has been implicated in single-strand breaks in DNA (13).

Biochemical modifications induced by 4-HC can either inhibit cellular proliferation until the damage is repaired or can promote initiation of an active cell death program; in the latter case the affected cell will be destroyed. The outcome of treatment will depend on three major factors: intracellular concentration of the drug, availability of the target and the ability to repair cytotoxic damage. Drug concentration in its turn is affected by the rates of uptake and efflux, drug's chemical stability and its detoxification by cellular enzymes.

One of the obvious means to increase effective intracellular drug concentration is the escalation of the administered dose, and this approach remains one of the most frequently used tactics in cancer chemotherapy. Unfortunately, chemotherapeutic agents are essentially toxins which affect normal tissues as well as malignant neoplasias, and toxic side effects can become unacceptable during and after drug treatment. Furthermore, even the maximum tolerated dose (MTD) of any drug gives just temporary improvement, since neoplastic cells usually become resistant to a particular class of cytotoxic compounds, so that subsequent treatments - however toxic to the patient - would no longer produce any deleterious effect on the tumor itself (14). Resistance to chemotherapy develops gradually and depends on alterations in genome functions (e.g. induction of MDR1 expression which leads to increased efflux of some drugs thus reducing their intracellular concentration, 15-17); in case of CP known mechanisms of resistance involve elevated levels of glutathione (18), increased activity of glutathione-S-transferase (19), aldehyde dehydrogenase (ALDH, 20) and gamma-glutamyl transpeptidase (21). Genetic regulation of resistance implies that genes involved can be studied and used either to block such resistance in tumor cells and thus make them susceptible to chemotherapy, or to protect normal cells against chemotherapeutic side effects. Obviously, increasing the level of resistance in normal cells would allow rapid dose escalation and increase the overall efficiency of chemotherapeutic treatment.

In advanced breast cancer high-dose chemotherapy is an effective strategy to significantly reduce tumor burden in cancer patients. Hyrnuik et al. (22, 23) observed direct correlation between dose intensity of CP and remission rate in advanced breast cancer; clearly, the higher the dose of initial chemotherapeutic challenge the better are chances of remission. CP dose increase is limited by side effects on bone marrow, uro-, cardio- and pulmonary toxicity as well as terato- and oncogenic effects (24); myelosuppression is the major problem since hematopoietic cells are extremely sensitive to the toxic effects of CP and other chemotherapeutic drugs (25). Severe leukopenia and granulocytopenia as a result of CP treatment increases patient's susceptibility to pathogens and opportunistic bacteria: infectious complications, including septicaemia, are common in patients after high dose of CP (26). Reconstruction of hematopoietic stem cell population after high-dose chemotherapy is realized by (ABMT) or (PBSCT), coupled with administration of hematopoietic growth factors. This approach permits significant (10-20 fold, 27) dose escalation with relatively low morbidity and considerable increase in disease-free survival (28). A serious drawback of high-dose chemotherapy in combination with ABMT/PBSCT is the cost of the procedure ($48,000 to $384,000, 29); considering that such treatment has to be administered repeatedly the overall economic impact in some cases becomes prohibitive (29, 30).

Obviously, increased resistance of hematopoietic stem cells to CP treatment would lead to reduction in both the morbidity rate and cost of the treatment since it would (1) allow rapid dose escalation without adverse effects on hematopoietic system, (2) reduce or even eliminate the risk of infectious complications after treatment, and (3) eliminate the need for repeated ABMT/PBSCT. In this project we propose to identify genetic elements which would provide cell protection from cytocidal

effects of CP and its derivatives. Such elements can then be used for gene therapy as well as for determination of mechanism(s) of resistance against CP and its analogues. Currently, ALDH has been suggested for gene therapy application as a potential chemoprotectant against CP: transfection of ALDH was reported to provide several-fold increase in resistance to CP anlogues (31). Whether ALDH-conferred modest resistance to CP will be applicable and/or sufficient for hematopoetic cell chemoprotection remains to be established.

Genetic mechanisms of resistance can be analyzed by expression comparison or expression selection. In the former, genes that are differentially expressed in resistant versus sensitive cells can be identified through subtractive hybridization (32) or differential display (33) and such genes can then be tested for ability to confer resistance when overexpressed in breast cancer cells. This approach can be very productive when a major difference in expression of an important gene is evident (e.g. 34, 35). On the other hand, slight changes of regulatory gene expression, that cannot be detected by either method, can cause dramatic alterations of phenotype (e.g. 36). Such regulatory genes likely will be excluded from initial analysis, and ultimately there is a risk of screening differentially expressed genes that have nothing to do with resistant phenotype.

Alternatively, one can apply expression selection technique to *functionally* select cells that express a desired phenotype. In this case inhibition (attenuation) of a "sensitivity" gene or increased expression (augmentation) of a "resistance" gene will produce the same increased resistance to CP, although experimental techniques are very different. When searching for a "resistance" gene, a cDNA expression library is introduced into a cell population which is then subjected to selection (37,38). Adjusting selection conditions, one can ensure that only cells expressing a favorable phenotype will survive (if selection is with cytotoxic agent) or proliferate (if selection agent is cytostatic). These cells are expected to express an introduced gene(s), which promotes cell survival or proliferation (expression augmentation strategy).

To identify "sensitivity" genes which make the cell susceptible to the drug disruption of its expression is required. Random inactivation of expressed genes can be achieved by retrovirus-mediated insertional mutagenesis since integration of retroviral DNA occurs primarily within active genes (39) thus interrupting transcription from the gene. After retroviral infection infected population can be selected for drug-resistance and surviving colonies analyzed. However, broad application of this technique is limited by the fact that both alleles have to be mutated to ensure changes in phenotype. Assuming that there are 100,000 actively expressed genes per cell (40) and that both alleles are expressed, at least 10^{10} infected cells with two copies of proviral DNA per genome have to be screened to identify a single clone that has both alleles inactivated.

An elegant way to address the problem of the second allele through posttranscriptional inhibition of gene expression is offered by the Genetic Suppressor Element (GSE) method (41, 42). The key component of this system is a library of normalized cDNA fragments cloned in random orientation into an expression vector (42). When expressed in target cells these cDNA fragments can act as antisense RNA or as dominant negative peptides, blocking either translation or function of the protein regardless of how many alleles are active (42).

Combination of augmentation and attenuation approaches is presumed to provide full spectrum of genes involved in development of resistant phenotype.

Body

Experimental methods, assumptions and procedures

General description of experimental design.

The broad aim of the project is to change the genetic environment of CP-sensitive cells and to determine which changes confer resistance to CP. The experimental approach consists of two parts: (a) identification of genes that have to be *repressed* for the cell to become resistant and (b) identification of genes that induce CP resistance when *expressed.* Since CP has to be activated by cytochrome P-450 in vivo we are using its in vitro active analogue mafisfamide (MA).

The first part of the project requires transduction of a GSE library into MA-sensitive cells and selection of actively growing clones after treatment MA. GSEs from these clones will also be individually tested for their ability to protect cells against MA. For this part of the project we use HT1080 cells which express ecotropic-receptor (NH1080/Eco).

The second part of the project involves transfection of MA-sensitive cells with a cDNA expression library and selection of cells that survive treatment with MA. Individual cDNA clones recovered from such cells have been tested separately for ability to confer resistance to MA. The cDNA expression library that we employ in this study is constructed in shuttle vector pcDNA3.1, which contains the SV40 replication initiation site (origin). Shuttle vectors can persist as extrachromosomal plasmids (episomes) in mammalian cells and can be quickly and efficiently rescued using the Hirt extraction procedure (43). Episomal persistence of SV40-based plasmids requires expression of large T antigen (TAg) of SV40, which is provided *in trans*. Temperature-sensitive mutant of tsTAg prevents overreplication of transfected plasmids at 37°C (nonpermissive conditions) and supports it when cells are shifted to permissive temperature (33°C); 5 day incubation at 33°C allows accumulation of plasmids and greatly facilitates their isolation by the Hirt procedure. For this part of the project we use Cos-ts-2 cells which express tsTAg.

Results and discussion

GSE approach.

Introduction of normalized library of random cDNA fragments cloned in retroviral expression vector LNCX into HT1080 cell line and selection for GSEs conferring resistance to CP derivative mafosfamide.

Normalized library of short (250-400 bp) fragments of cDNA cloned in retroviral expression vector LNCX (44), as well as HT1080/Eco cell line have been provided by Dr.Igor Roninson. LNCX contains a dominant selectable marker (neomycin phosphotransferase) which allows efficient selection of infected cells in G418-containing medium (44). This library has been transfected by standard calcium-phosphate co-precipitation procedure into ecotropic packaging cell line BOSC 23 (40) to generate infectious viral particles which were used to infect HT1080/Eco cells. Transduction of LNCX without an insert has been used as a control. Efficiency of transduction was determined by (a) infection and G418 selection of NIH 3T3 fibroblasts to evaluate viral titer; and by (b) selection of a defined

number of infected HT1080/Eco in G418-containing media. Number of G418-resistant clones obtained versus plating efficiency served as a measure of transduction efficacy. In our experiments 25-45% of the HT1080/Eco cells can be infected by this method; for library selection $3x10^7$ infected cells were used to ensure complete representation of the library (its complexity is estimated to be approx. 10^7).

HT1080/Eco cells were treated with MA for 24 hr to simulate continuous drug application. Surviving colonies after the first round of selection were expanded, and their genomic DNA was used for PCR-mediated recovery of inserts. These inserts were directionally cloned into LNCX vector, and this second-order library was used for an additional round of selection.

Single-base PCR sequencing reactions will be performed and different clones identified. Non-identical individual clones will be tested for their ability to confer resistance to MA through BOSC 23 transfection, infection of the target cells and selection with MA. Different stages of our experimental strategy have been tested in pilot experiments with satisfactory results: mafosfamide selection of library-transduced HT1080/ETR cells gave a significant number of surviving colonies while control population was totally eliminated.

Initial results with selection of HT1080/Eco cells with MA are presented in Fig. 1; protective effect of GSE library has been apparent. However, after PCR-mediated recovery of inserts, cloning into LNCX vector and re-transduction of the second order library (see Fig. 2) we could not obtain any protective effect against MA. Three attempts were made to duplicate protective effect using the second order library but no significant protection was achieved (data not shown). Since we have the same problem with another project, which also involves GSE methodology, we believe that there are common reasons for the loss of protection at the second order library phase; in our opinion, the most probable cause is inefficient recovery of the inserts from genomic DNA of surviving cells after the first round of selection. There are several arguments which support this hypothesis: (1) location of primers used for PCR favors amplification of short DNA fragments (average size of amplified fragment approx. 220 bp plus 126 bp of vector sequences; compare this with average size (300 bp) of cloned fragments in the initial library); within this size range the shorter the fragment the better its yield and longer fragments will be eventually lost from the population. (2) Directional cloning in the original scheme was achieved by elimination of the upstream ClaI site (see Fig.2); for this purpose upstream primer contained a mismatched nucleotide and was located within the adaptor sequence. This sequence proved to be extremely inconvenient for priming in the first place and, in combination with mismatched nucleotide within ClaI site, produced a significant number of incorrect junctions vector-insert: 12 out of 16 sequenced clones lost a part of upstream adaptor sequence. Since adaptor provides for translation initiation in all three reading frames this type of mutation can have significant effect on the translation of GSEs. We have redesigned the primers in such a way that the average size of PCR product is approx. 800 bp (565 bp of which are vector sequences) and the sequences of vector-insert junctions are preserved during cloning; using these primers PCR product from the initial library is of the same size as that from the second order library and no rearrangements of upstream junction/adaptor has been observed in eight sequenced clones. We believe that new selection with the second order library constructed through thus modified approach will be successful.

Due to the problems with the second order library we are delayed in our progress by approx. 5 months (currently our progress corresponds to Objective 1, Task 3 of SoW).

Full length cDNA selection.

Introduction of full length cDNA expression library into CP-sensitive cells Cos-ts-2 and selection for cDNA clones that protect cells from CP analogue Mafosfamide (MA) .

cDNA expression library from human fetal brain is of the highest possible complexity [estimated complexity of brain mRNA exceeds 10^5 (46), thus brain cDNA library has nearly all transcribed human genes (40)], giving us a good chance of successful selection; this library has been purchased from Invitrogen (San Diego, CA, USA). Shuttle vector pcDNA3.1, used for directional construction of the library, contains a dominant selectable marker (neomycin phosphotransferase), which allows selection in G418-containing medium, and SV40 origin, which allows replication in the presence of SV40 large TAg. Temperature-sensitive tsTAg is supplied *in trans* (47). We have used the cDNA library for expression selection of genes conferring resistance to the replication inhibitor aphidicolin with good results. Transfection is done using the cationic lipid reagent LipofectAmine (GibcoBRL) according to the manufacturer's specifications. Efficiency of transfection is determined by selection of a defined number of transfected cells in G418-containing media. Number of G418-resistant clones versus plating efficiency serves as a measure of transfection efficiency; in preliminary experiments up to 25% of transfected cells expressed b-galactosidase. For selection we used $8x10^6$ transfected cells to ensure complete representation of the library, which contains $2x10^6$ primary clones. Cells transfected with vector plasmid served as a control.

Selection of transfected Cos-ts-2 cells was done as determined in preliminary experiments: 24 hr exposure to 50 uM mafosfamide eliminated approx. 99.9% of parental cells. Actively growing colonies are expanded and incubated for 120 hr at 33°C to allow for overreplication of surviving plasmids, which are then isolated by the Hirt procedure and used for the second round of selection.

Initial results with selection of Cos-ts-2 cells with MA are presented in Fig. 3. As in the case of GSE library, protective effect of full length cDNA expression library is also evident. We have extracted plasmids from the surviving population and reintroduced them into Cos-ts-2 cells. Cells transfected with the second order library will be placed under selection shortly.

Individual cellular clones obtained after the first selection with MA have been expanded and re-tested (Fig. 4): the majority of them has been resistant to significantly higher doses of MA than those used for selection (75 uM versus 50 uM). Recovery of the plasmids and their analysis are underway.

Following the second round, plasmids will be recovered from individual cellular clones as before and different plasmids will be identified by colony hybridization and PCR sequencing. Non-identical plasmids will be individually transfected into Cos-ts-2 cells, which will then be tested for their growth after treatment with MA.

This part of the project has been initiated as complementary to the GSE approach after the approval of the award; due to this reason there is no corresponding entry in the SoW. The choice of cell line was determined by convenient regulation of episomal plasmid persistence; since recovered functionally active genes would have to be confirmed in hematopoetic human cell lines, e.g. K562, for both parts of the project we decided to perform primary screens in existing cell lines while developing corresponding counterparts based on K562.

Conclusions

1. Both attenuation and augmentation approach indicate that resistance to MA is a complex process influenced by several mechanisms which can be analyzed by the proposed methods.
2. Resistance to MA can develop both by repression and by activation of gene expression.

Although some technical adjustments to the proposed procedure proved to be necessary, results obtained so far can be considered as highly promising.

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Appendices

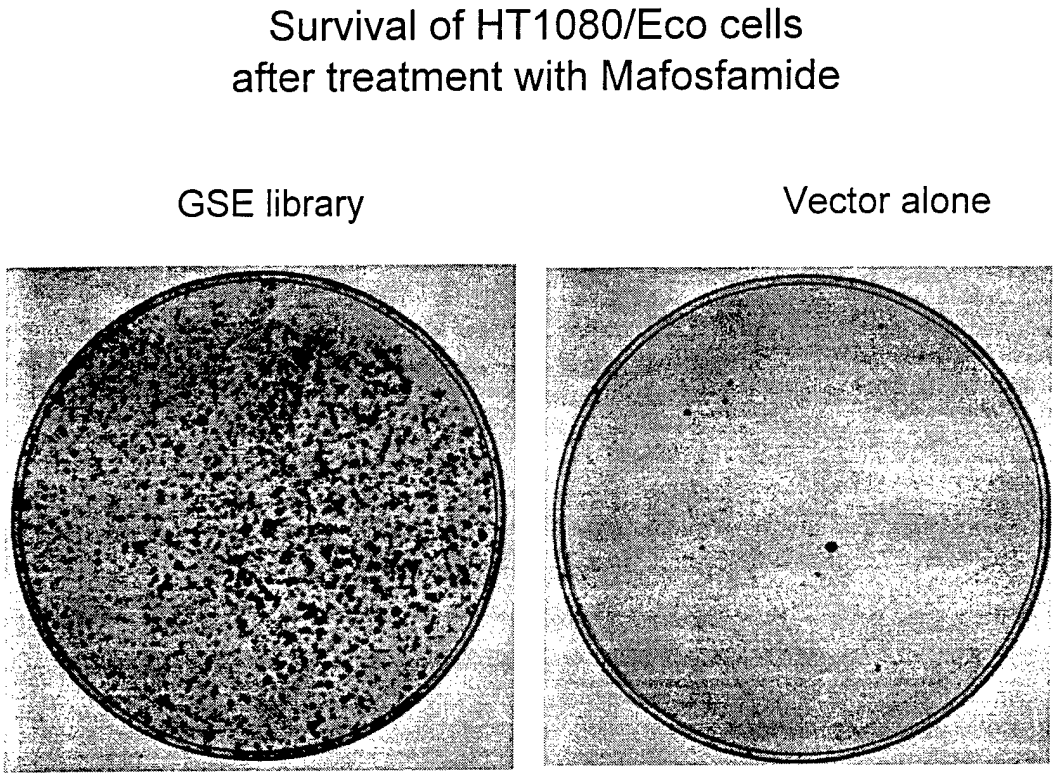

Fig. 1. HT1080/Eco cells were transduced either with GSE library or with vector alone. Efficiency of transduction - approx. 40% in each case. Selection conditions: 10^6 cells per p150, 25 uM Mafosfamide, 24 hr.

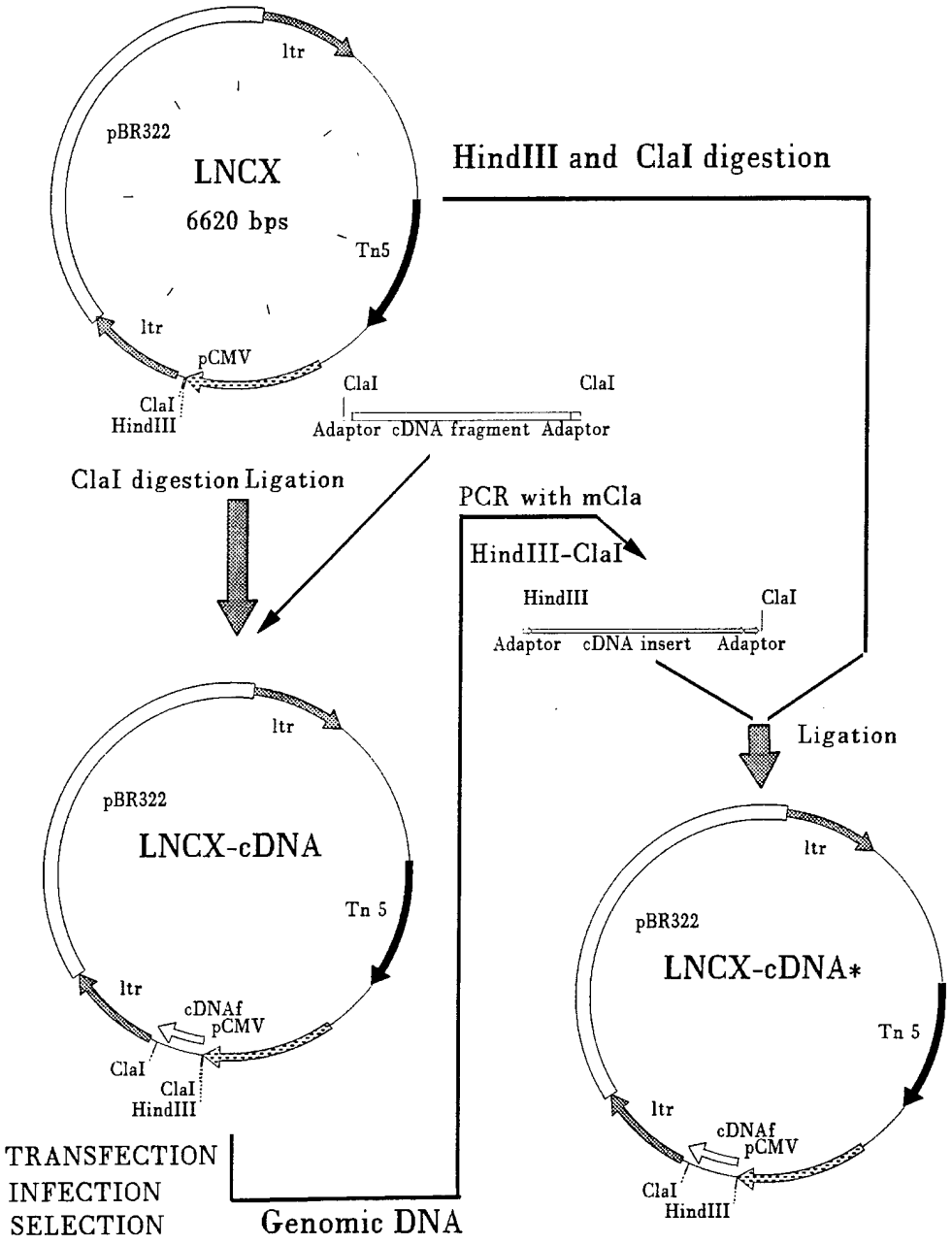

Fig.2. General scheme of cloning. Short fragments of cDNA are cloned in random orientation into ClaI site in LNCX vector. Resulting library is transducted into target cells, which are then selected for a desired phenotype. Genomic DNA from surviving population is used to recover inserts by PCR. Upstream primer (mCla) has mutated ClaI site, so that recloning is done through ClaI-HindIII

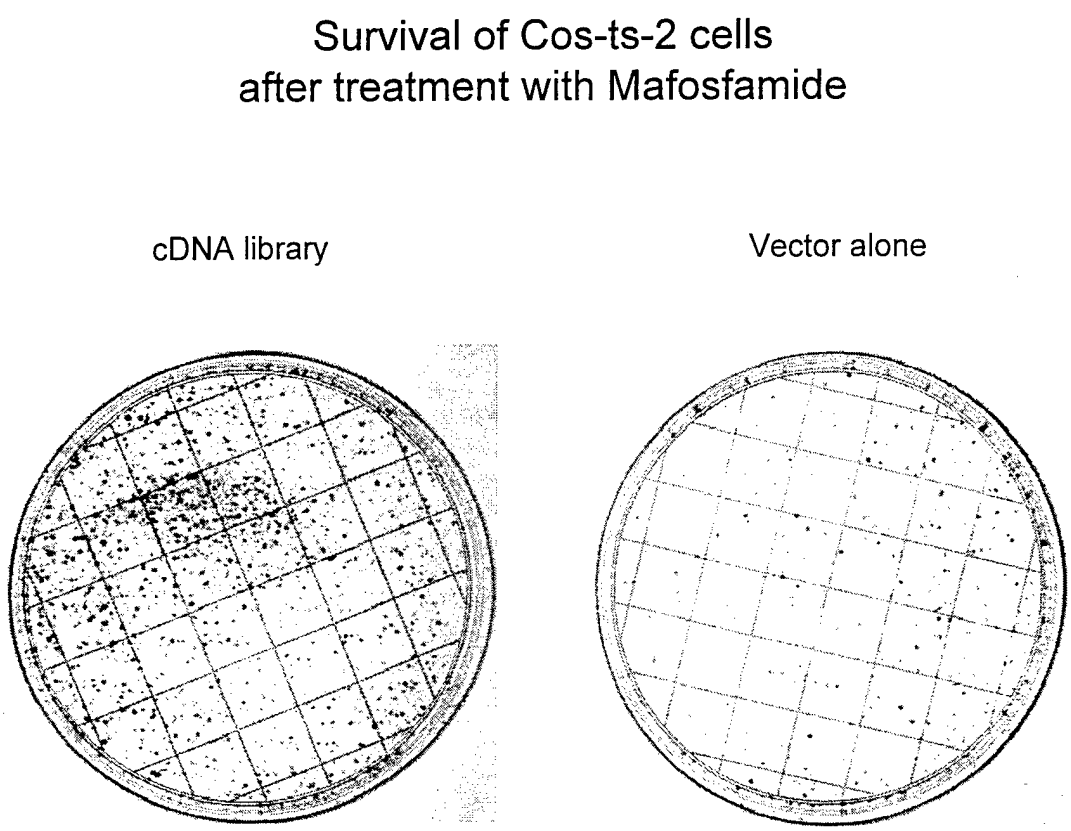

Fig. 3. Cos-ts-2 cells were transfected either with cDNA library or with vector alone. Efficiency of transfection - approx. 22% in each case. Selection conditions: 10^6 cells per p150, 50 uM Mafosfamide, 24 hr.

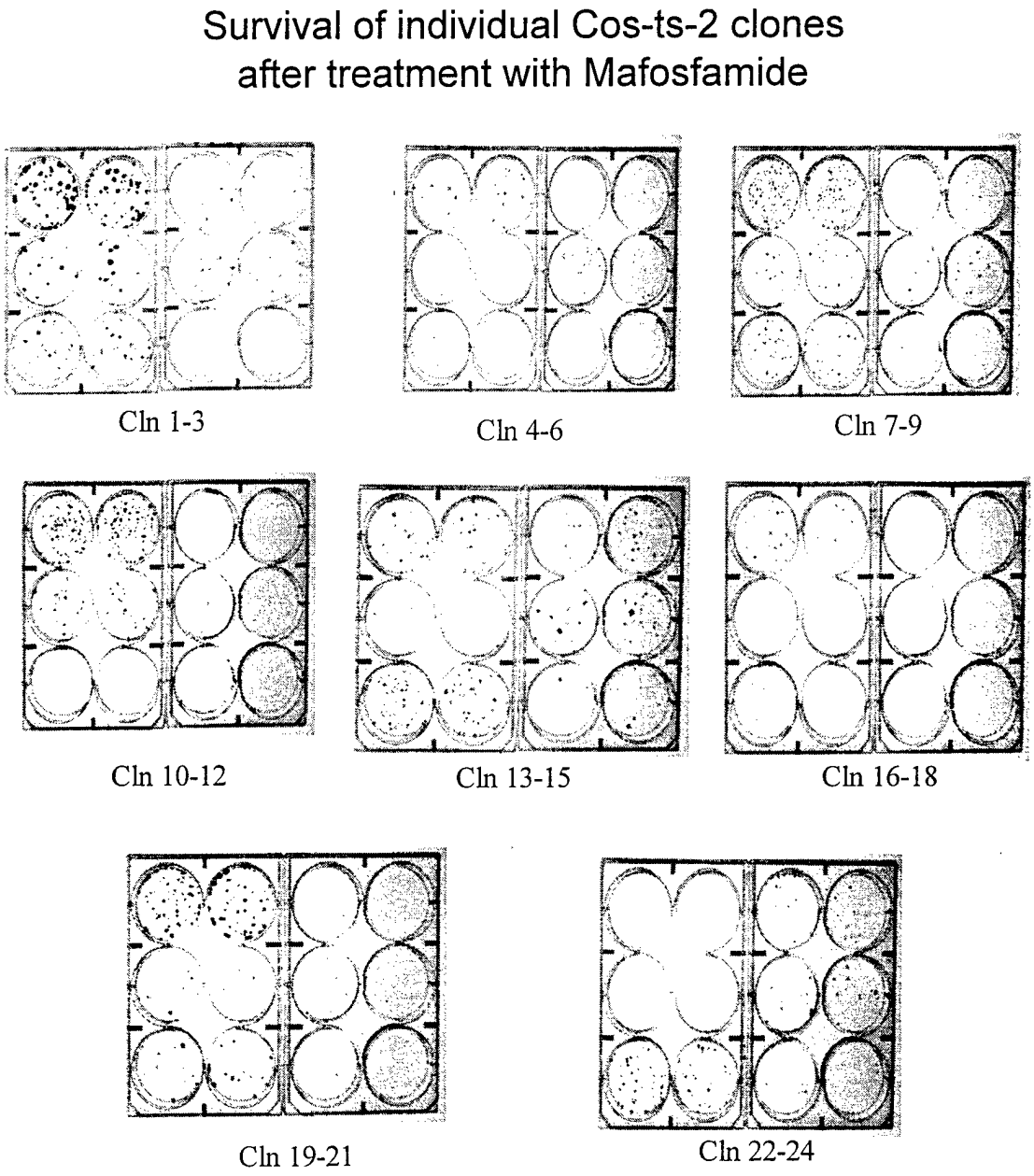

Fig.4. Individual surviving Cos-ts-2 clones after the first round of selection were expanded and re-tested for resistance to Mafosfamide. Selection conditions: 10^4 cells per well. 50 uM Mafosfamide, 24 hr.